

Influence of the renin-angiotensin system on epidermal growth factor expression in normal and cyclosporine-treated rat kidney

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Influence of the renin-angiotensin system on epidermal growth factor expression in normal and cyclosporine-treated rat kidney.

Background. Epidermal growth factor (EGF) plays an important role in renal tubular regeneration after ischemic injury in kidney. The present study reports the association between the renin-angiotensin system (RAS) and EGF, and the effect of angiotensin II blockade with losartan (LSRT) on EGF expression in an experimental model of chronic cyclosporine (CsA) nephrotoxicity in rats.

Methods. Two separate experiments were performed. In the first experiment, rats on the normal-salt diet (NSD; 0.3%) or low-salt diet (LSD; 0.05%) were treated with or without LSRT for four weeks. In the second experiment, rats on the NSD or LSD were given vehicle (VH group, olive oil, 1 mg/kg per day) or CsA (15 mg/kg per day) or CsA (15 mg/kg per day) plus LSRT (100 mg/L per day). Renal function, histopathology, TUNEL staining, plasma renin activity (PRA), and the expression of renin and EGF were studied.

Results. Normal rats on the LSD showed significantly increased EGF expression (cortex, 2.6-fold; medulla, 1.7-fold) and significantly decreased EGF expression with the LSRT treatment compared with the rats treated with the NSD (cortex, 74.8 vs. 10%; medulla, 22.5 vs. 5%). In contrast, the CsA-treated rats on the LSD had a significantly lower EGF expression (cortex, 98 vs. 53%; medulla, 94 vs. 14%); however, concomitant administration of LSRT increased the EGF expression (cortex, 91- vs. 3.8-fold; medulla, 19- vs. 2.4-fold) compared with the rats on the NSD. In the normal and CsA-treated LSD rats, EGF expression was well correlated with PRA. In addition, EGF expression was well correlated with the interstitial fibrosis score ($r = 0.664$, $P < 0.01$) or number of TUNEL-positive cells ($r = 0.822$, $P < 0.01$) in CsA-treated LSD rats.

Conclusions. These results suggest that angiotensin II blockade with LSRT decreases EGF expression in normal rats on the LSD, but it protects EGF expression in CsA-induced nephrotoxicity. This finding provides a new perspective on the renoprotection of angiotensin II blockade in chronic CsA nephrotoxicity.

Key words: losartan, nephrotoxicity, progressive renal disease, interstitial fibrosis, tubular atrophy, tubular regeneration, ischemic injury.

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Chronic cyclosporine (CsA)-induced nephrotoxicity is characterized by progressive renal failure and irreversible renal striped interstitial fibrosis, tubular atrophy, and hyalinosis of the afferent arteriole [1]. The precise mechanism of chronic CsA nephrotoxicity is not well understood, but has been attributed to sustained afferent arteriolar vasoconstriction and arteriopathy [2, 3].

The lack of an animal model of chronic CsA nephrotoxicity has hampered the study of the mechanisms of renal damage. However, a reproducible animal model of chronic CsA nephrotoxicity was established from the observation that sodium depletion exacerbates CsA nephrotoxicity [4, 5]. In this model, CsA treatment in rats on a low-salt diet (LSD) induced a histologic feature similar to that described in patients on long-term CsA therapy [6]. Salt depletion activates the renin-angiotensin system (RAS), which has been implicated in the changes in renal hemodynamics and function that follow CsA administration [7]. Furthermore, angiotensin-converting enzyme inhibitor or angiotensin II (Ang II) receptor antagonist is effective in preventing interstitial fibrosis [8] by inhibiting the expression of intrarenal transforming growth factor- β 1 (TGF- β 1) [9].

Epidermal growth factor (EGF) is a 53-amino acid (6.2 kD) polypeptide that stimulates the proliferation and differentiation of epithelial cells as well as being mitogenic for various other cell types in culture [10, 11]. In addition, EGF is thought to be important in the maintenance of renal tubule integrity and in the tubular regenerative response to injury [12, 13]. Animal studies show that EGF accelerates renal tubular regeneration in rat models of acute renal failure [14], whereas treatment with anti-EGF antibody inhibits tubular cell proliferation during compensatory renal growth [15].

Epidermal growth factor expression has been well studied in clinical and experimental transplantation. Jorgensen et al reported that urinary excretion of EGF in living human kidney recipients is 40% lower than in donor

kidneys, and urinary expression is well correlated with compensatory renal growth, suggesting decreased urinary EGF excretion by ischemic or CsA-induced toxicity [16]. Di Paolo et al reported that the renal expression and the urinary concentration of the EGF signal are extremely weak or even absent in patients with acute rejection, acute tubular necrosis, and CsA nephrotoxicity [17]. Stein-Oakley et al reported that chronic renal allograft rejection shows decreased expression of EGF in a rat model [18]. These findings suggest that tubular damage by ischemia, nephrotoxic drugs, or rejection decreases EGF expression.

The association of EGF expression and RAS is well known from studies in vitro and in the developing kidney [19]. Angiotensin II potentiates the mitogenic action of EGF on proximal tubular cells [20], and the inhibition of angiotensin II type 1 (AT1) receptors decreases the renal expression of EGF in neonatal rats [21]. Based on these observations, we hypothesized that EGF expression may be influenced by RAS in chronic CsA nephrotoxicity. To test this hypothesis, two separate experiments were performed. First, we evaluated the difference in EGF expression of normal rats on a low-salt diet (LSD) or a normal-salt diet (NSD), and evaluated the influence of Ang II blockade on EGF expression. Second, EGF expression was evaluated in CsA-treated rats with an LSD or an NSD, and the influence of Ang II blockade on EGF expression was evaluated in normal and CsA-treated rat kidneys. Our study clearly demonstrates the influences of RAS on intrarenal EGF expression in rats with or without CsA treatment.

METHODS

Animals

Male Sprague-Dawley rats (Charles River, Wilmington, MA, USA), weighing 225 to 250 g, were housed in individual cases in a temperature- and light-controlled environment. They received the LSD (0.05% sodium; Teklad Premier, Madison, WI, USA) or NSD (0.4% sodium; Teklad Premier). NSD rats were pair fed with LSD animals.

Drugs

Cyclosporine provided by Sandoz Research (East Hanover, NJ, USA) was diluted in olive oil to a final concentration of 15 mg/mL. Losartan (LSRT), provided by Cardiovascular Diseases Research, DuPont-Merck Pharmaceutical (Wilmington, DE, USA) was dissolved in sterile water to a final concentration of 100 mg/L.

Experimental groups

Two separate experiments were conducted. The first experiment (protocol 1) was designed to evaluate the role of RAS in regulating EGF expression in normal rat kidney.

Protocol 1. (1) NSD rats received a 0.4% sodium content diet for four weeks ($N = 6$). (2) NSD-LSRT rats received a 0.4% sodium content diet plus LSRT (100 mg/L) in the drinking water for 28 days ($N = 6$). (3) LSD rats received a 0.05% sodium content diet for four weeks ($N = 6$). (4) LSD-LSRT rats received a 0.05% sodium content diet and LSRT (100 mg/L) in the drinking water for 28 days ($N = 6$).

Protocol 2. This study was designed to evaluate the role of RAS in regulating EGF expression in CsA-treated rat kidney and to examine the effect of blocking the AT1 receptor on EGF expression. The NSD or an LSD was administered to the each group. (1) The vehicle (VH) group of rats received a daily subcutaneous injection of olive oil, 1 mg/kg for four weeks ($N = 6$). (2) CsA rats received a daily subcutaneous injection of CsA (15 mg/kg) for four weeks ($N = 6$). (3) CsA + LSRT rats received a daily subcutaneous injection of CsA (15 mg/kg) and LSRT (100 mg/L) was added to the drinking water for four weeks ($N = 6$).

This dosage and route of administration for LSRT were selected because they have been previously shown to blockade Ang II receptors significantly [22].

Basic protocol

After one week of the animals on the NSD or LSD, weight-matched rats were randomly assigned to the different treatment groups. Daily body weights were recorded. After each treatment period, the systolic blood pressure was measured with a plethysmography using a tail manometer-tachometer system (BP-2000; Visitech Systems, Apex, NC, USA), and 24-hour urine samples were collected in metabolic cages (Nalge Co., Rochester, NY, USA). The following day, animals were anesthetized with ketamine, and a blood sample and tissue specimens were obtained.

Functional studies

Urinary and serum creatinine was measured by a Cobas autoanalyzer (Roche Diagnostics, Div. Hoffman-La Roche Inc., Nutley, NJ, USA). The creatinine clearance (C_{Cr}) was calculated using a standard formula. The whole-blood CsA concentrations were measured by monoclonal radioimmunoassay (Incstar Co., Stillwater, MN, USA). Plasma renin activity (PRA) was measured by radioimmunoassay (Angiotensin I-Biotecx radioimmunoassay; Biotecx, Friendswood, TX, USA).

Histology

These histologic findings were evaluated as previously described [23]. Tubulointerstitial fibrosis (TIF) was estimated semiquantitatively using a color image analyzer (Mustek Paragon 800 SP, Macintosh PowerPC 7100, NIH Image version 1.5) by counting the percentage of injured areas per field of cortex and medulla. Scores of 0 to 3+

were given as follows: score 0 = normal interstitium; score 0.5 = <5%; score 1.0 = 5 to 15%; score 1.5 = 16 to 25%; score 2.0 = 26 to 35%; score 2.5 = 36 to 45%; and score 3.0 = >45%.

Tdt-mediated dUTP-biotin nick end-labeling (TUNEL) method

Cells undergoing apoptosis were identified by the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD, USA). The sections were dewaxed and treated with proteinase K and then incubated with equilibration buffer in a humidified chamber for 10 minutes at room temperature, followed by incubation with working-strength Tdt enzyme solution in a humidified chamber at 37°C for two hours. The reaction was terminated by incubation in working-strength stop/wash buffer for 30 minutes at 37°C. After being rinsed with phosphate-buffered saline (PBS), the sections were incubated with anti-digoxigenin peroxidase in a humidified chamber for 30 minutes at room temperature. Sections were then incubated with diaminobenzidine and 0.01% H₂O₂ for five minutes at room temperature. After being rinsed with PBS, the sections were counterstained with hematoxylin and examined by light microscopy. To quantitate TUNEL-positive cells, 16 fields of outer medulla were randomly selected in each section and observed under a microscope (magnification ×400). The number of apoptotic cells was expressed per 1000 of the total tubular cells in each section.

Immunoblotting for EGF

Kidneys were homogenized in RIPA [10 mmol/L Tris Cl, pH 7.6, 150 mmol/L NaCl, 1% (wt/vol) sodium deoxycholate, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) sodium dodecyl sulfate, 1% (vol/vol) aprotinin, 2 mmol/L Na₃VO₄, and freshly added leupeptin (1 µg/mL), pepstatin (1 µg/mL), and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)]. Homogenates were centrifuged at 16,000 × *g* for 15 minutes at 4°C, and protein concentrations were determined using a protein microassay of Bradford method (Bio-Rad, Hercules, CA, USA). Homogenates were heated at 94°C for five minutes. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 15% polyacrylamide gels and were electroblotted onto Bio-Blot nitrocellulose (Costar, Cambridge, MA, USA). Nonspecific binding was blocked by incubating the blots for one hour in 5% (wt/vol) nonfat milk. Epidermal growth factor was detected by incubating for one hour with a polyclonal anti-EGF antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:1000. Primary antibody incubation was followed by six washes of Tris-buffered saline with 0.005% Tween 20 (TBS-T). The blot was then incubated with secondary antibody [goat-anti-rabbit IgG-horseradish peroxidase (HRP) conjugate at

Table 1. Changes in cyclosporine A (CsA) whole blood level, systolic blood pressure (SBP), serum creatinine (S_{Cr}), and creatinine clearance (C_{Cr}) in the different experimental groups

	CsA level ng/mL	SBP mm Hg	S _{Cr} mg/dL	C _{Cr} mL/min/100 g
NSD				
VH		138 ± 11	0.55 ± 0.02	0.55 ± 0.02
CsA	3826 ± 310	157 ± 21 ^a	0.80 ± 0.09 ^a	0.39 ± 0.03 ^a
CsA + LSRT	4273 ± 170	139 ± 12 ^b	0.85 ± 0.11 ^a	0.38 ± 0.12 ^a
LSD				
VH		127 ± 5	0.57 ± 0.02	0.61 ± 0.08
CsA	3100 ± 380	128 ± 7	1.02 ± 0.06 ^a	0.31 ± 0.06 ^a
CsA + LSRT	3260 ± 520	110 ± 4 ^{ab}	1.05 ± 0.17 ^a	0.30 ± 0.05 ^a

Data are presented as mean values ± SEM of six rats. Abbreviations are: NSD, normal salt diet; VH, vehicle; LSRT, losartan.

^a *P* < 0.05 vs. VH

^b *P* < 0.05 vs. CsA

1:10,000; Bio-Rad] for 30 minutes. Antibody-reactive protein was detected using enhanced chemiluminescence (Amersham Life Science, Little Chalfont, Buckinghamshire, UK). Densitometric analysis was performed using Gelexpert software (Nucleotech Corp., San Carlos, CA, USA). Optical densities (mean ± SEM) were obtained after three determinations for each band.

Immunohistochemistry of EGF and renin

Sections were dewaxed. Before incubation with primary antibody, the sections were blocked with normal rabbit serum diluted 1:10 in 0.3% bovine serum albumin (BSA) for 30 minutes and incubated for two hours at 4°C in mouse antiserum against EGF (Biomedical Technologies Inc., Stoughton, MA, USA) diluted 1:200 in BSA. The sections were rinsed in TBS and incubated for 30 minutes in peroxidase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Sections were incubated with a mixture of 0.05% 3,3'-diaminobenzidine and 0.01% H₂O₂ for five minutes at room temperature, washed with Tris-HCl buffer, counterstained with hematoxylin, and examined by light microscopy. The method for renin immunohistochemistry was same as EGF immunohistochemistry. The number of renin-positive glomeruli was counted per 50 glomeruli.

Statistical analysis

Data reported are mean ± SEM, and all statistical analyses were calculated with SYSTAT for Macintosh version 5.2 (SYSTAT Inc., Chicago, IL, USA). Comparisons between groups were done by analysis of variance (Kruskal-Wallis test followed by Tukey or Dunnet test) or unpaired *t* test. Nonparametric (Spearman) correlation coefficient was calculated. The level of statistical significance was *P* < 0.05.

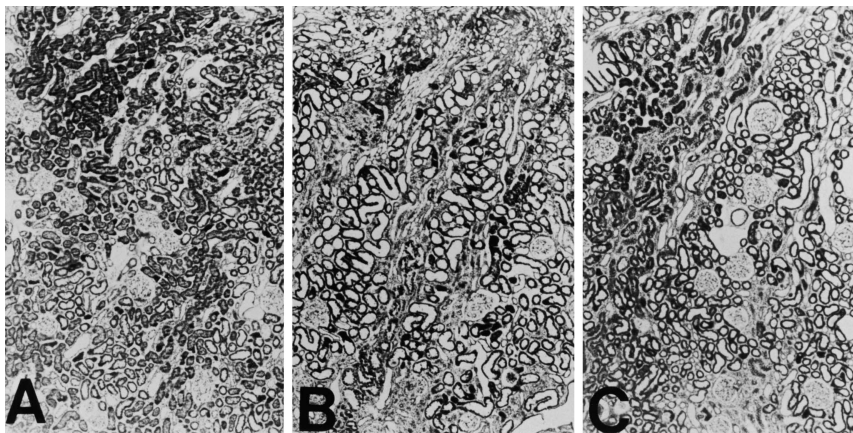


Fig. 1. Effect of angiotensin II blockade with losartan (LSRT) on cyclosporine A (CsA)-induced chronic nephropathy. (A) Vehicle-treated rat kidney. (B) The sodium-depleted animal treated with CsA. There is a diffuse interstitial fibrosis, mononuclear cell infiltrate, and tubular atrophy. (C) CsA and LSRT-treated rat kidney. Note the decrease in interstitial fibrosis and tubular atrophy. Trichrome stain, magnification $\times 100$.

RESULTS

Blood pressure and functional assessment

Table 1 shows the blood pressure and renal function in the experimental groups. At four weeks, systolic blood pressure was increased in the CsA group with an NSD, but there was no significant change in the CsA group with an LSD. The addition of LSRT decreased systolic blood pressure compared with the VH group on the NSD and LSD. The CsA group with an NSD and an LSD showed a significant decrease in C_{Cr} and a significant increase in serum creatinine compared with the VH group ($P < 0.05$, respectively). However, there were no significant differences in C_{Cr} between CsA and CsA + LSRT groups with NSD or LSD.

Histologic assessment

Low salt diet rats treated with CsA had characteristic morphologic findings that were usually evident at four weeks and were similar to the chronic human CsA renal lesions (Fig. 1B). Focal TIF, tubular atrophy, and mononuclear inflammatory cell infiltration were observed. The concurrent administration of LSRT dramatically decreased TIF (Fig. 1C). When the extent of changes in TIF was graded with a 0 to 3+ semiquantitative score, there were no differences in the TIF scores among VH (0.17 ± 0.14), CsA (0.21 ± 0.19), and CsA + LSRT groups (0.19 ± 0.18) on a NSD. However, in the LSD group, there was a significant increase in TIF score in the CsA group compared with the VH group (2.07 ± 0.56 vs. 0.19 ± 0.13 , $P < 0.05$). The concomitant administration of LSRT decreased the TIF score in comparison with the CsA group (1.25 ± 0.25 ; Fig. 2).

Apoptotic cell death in CsA-treated rats

Figure 3 shows TUNEL-staining in chronic CsA nephrotoxic rats on the LSD. In the VH group, TUNEL-positive cells were rarely observed. The number of TUNEL-positive cells increased with CsA treatment, but decreased

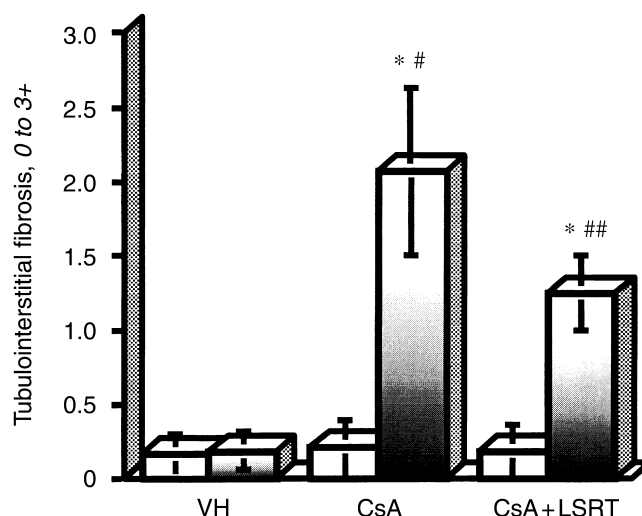


Fig. 2. Comparison of tubulointerstitial fibrosis (TIF) between the normal-salt diet (NSD; □) and the low-salt diet (LSD; ■) ingesting vehicle (VH) or cyclosporine A (CsA)-treated rats. In the LSD rats, there was a significant increase in TIF score in CsA-treated rats and a dramatic decrease of TIF with LSRT treatment in CsA-treated rats. In contrast, there was no significant difference of TIF in rats with the NSD. * $P < 0.05$ vs. NSD; # $P < 0.05$ vs. VH; ## $P < 0.05$ vs. CsA.

with LSRT treatment. Figure 4 shows the comparison of TUNEL-positive cells in the CsA-treated rats on the NSD and LSD. In the NSD group, there was no significant increase in the number of TUNEL-positive cells with CsA treatment compared with the VH group (4.8 ± 2.5 vs. 2.2 ± 1.6 , $P > 0.05$), and LSRT treatment did not affect apoptotic cell death (3.9 ± 1.7). In the LSD group, CsA treatment increased the number of TUNEL-positive cells compared with the VH group (52.1 ± 15.5 vs. 2.3 ± 0.9 , $P < 0.05$), and concomitant administration of LSRT significantly decreased the number of TUNEL-positive cells (15.6 ± 3.0) as compared with the CsA group ($P < 0.05$).

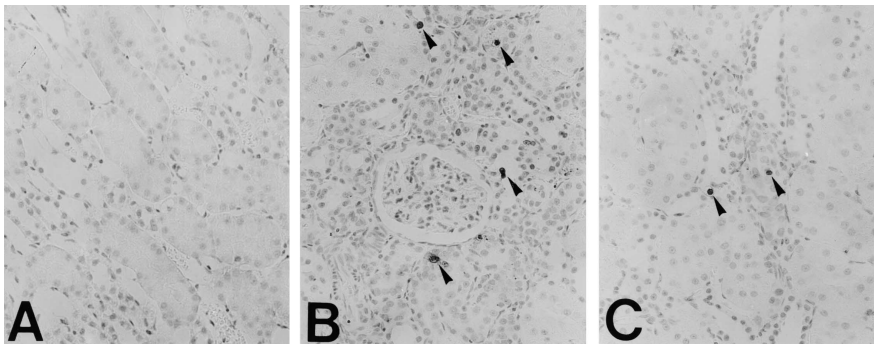


Fig. 3. Apoptotic cell death in CsA-treated rats with LSD. Photomicrographs show apoptosis as detected by the TUNEL assay in VH (A), CsA (B), and CsA + LSRT (C) groups with the LSD. Note the marked increase in TUNEL-positive cells (arrowheads) in the CsA-treated rats and decrease in TUNEL-positive cells in CsA + LSRT groups (magnification $\times 200$).

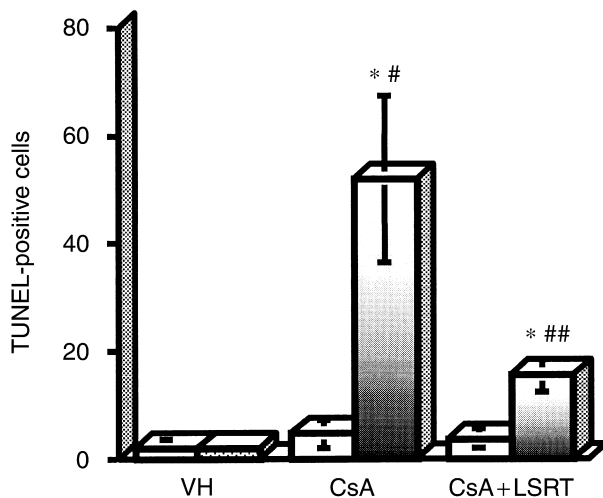


Fig. 4. Comparison of TUNEL-positive cells of vehicle and CsA-treated rats between those on the NSD (□) and the LSD (▨). There was no significant change of TUNEL-positive cells in CsA-treated rat kidney with NSD, but there was a significant increase of TUNEL-positive cells in CsA-treated rats with the LSD. Concomitant administration of CsA and LSRT decreased TUNEL-positive cells in rats with LSD. ^{*} $P < 0.05$ vs. NSD; [#] $P < 0.05$ vs. VH; ^{##} $P < 0.05$ vs. CsA.

Activity of renin-angiotensin system in CsA-treated rats

Figure 5 shows the intrarenal expression of renin and the results of quantitative analysis of renin-positive glomeruli in kidneys of CsA-treated rats on the NSD and LSD. Intrarenal expression of renin was minimal in the VH group, but its immunoreactivity and number of renin-positive glomeruli were increased in the kidneys with CsA treatment. Quantitative analysis showed a significant increase in the number of renin-positive glomeruli in rats on the LSD compared with the NSD in the VH group (11 ± 3 vs. 6 ± 2 , $P < 0.05$). CsA treatment increased renin-positive glomeruli compared with the VH group in both the NSD and LSD, but a greater increase was observed in the LSD group (25 ± 2 vs. 15 ± 3 , $P < 0.05$).

In the VH group, PRA was significantly increased in the LSD group compared with the NSD group ($13.8 \pm$

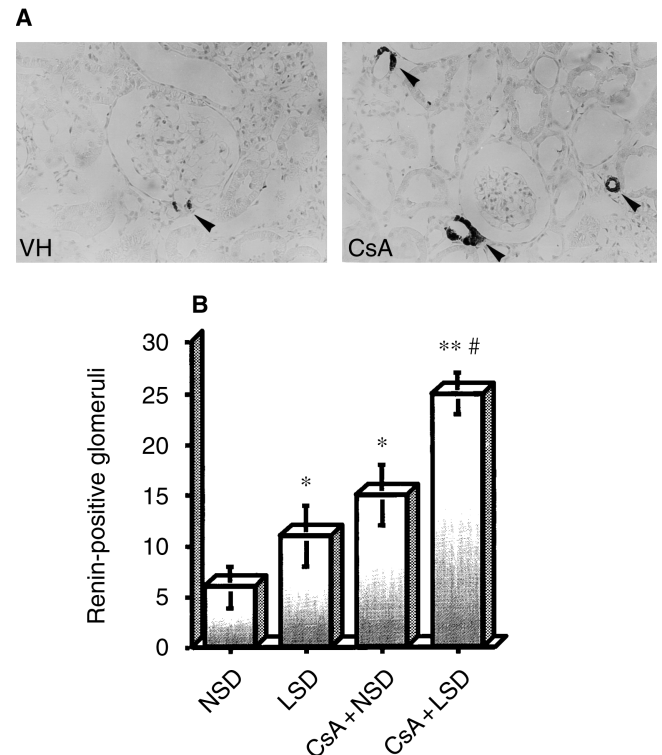


Fig. 5. Intrarenal renin expression in vehicle and CsA-treated rats. (A) Immunohistochemistry of renin in VH and CsA groups. In the VH group, there was minimal immunoreactivity of renin, but its immunoreactivity was strongly increased in the CsA-treated rat kidney (magnification $\times 200$). (B) Quantitative analysis of renin-producing glomeruli in the VH or CsA-treated NSD and LSD rats. Note the significant increase of renin-producing glomeruli in rats on the LSD compared with the NSD, and the increase of renin-producing glomeruli in CsA-treated LSD rats compared with NSD rats. ^{*} $P < 0.05$ vs. NSD; ^{**} $P < 0.05$ vs. CsA + NSD; [#] $P < 0.05$ vs. LSD.

5.6 ± 1.7 , $P < 0.05$). CsA treatment increased PRA in both the NSD (9.3 ± 2.8) and the LSD (38.8 ± 6.2) compared with the VH group ($P < 0.05$, respectively). The concomitant administration of LSRT significantly increased PRA (26.3 ± 5.0) in the NSD, but there was a dramatic decrease of RPA in the LSD (7.0 ± 1.8) as compared with the CsA ($P < 0.05$, respectively; Fig. 6).

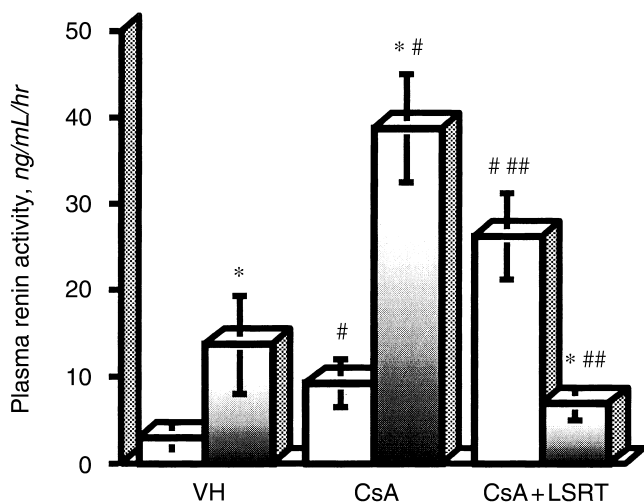


Fig. 6. Comparison of plasma renin activity (PRA) between the NSD (□) and the LSD (■) CsA-treated rats. Quantitative analysis of renin-positive glomeruli demonstrates a significant increase in the LSD as compared with the NSD. CsA treatment also increased intrarenal expression of renin, and this increase was more definite in the LSD compared with NSD. * $P < 0.05$ vs. NSD; # $P < 0.05$ vs. VH; ## $P < 0.05$ vs. CsA.

Influence of the renin-angiotensin system on EGF expression in normal rats

The immunoblot and optical densities of EGF are shown in Figure 7. After four weeks on the LSD, EGF expression was significantly increased (cortex, 40 ± 3 vs. 103 ± 8 ; medulla, 77 ± 15 vs. 151 ± 19 , $P < 0.05$, respectively) compared with the rat kidney with the NSD (Fig. 7). A concomitant administration of LSRT did not affect EGF expression in the NSD, but there was a significant decrease of EGF expression in rats with the LSD (cortex, 103 ± 8 vs. 26 ± 5 ; medulla, 151 ± 19 vs. 117 ± 16 , $P < 0.05$, respectively).

Influence of the renin-angiotensin system on EGF expression in CsA-treated rats

The immunoblot and optical density of EGF are shown in Figure 8. Immunoblotting for EGF protein provided a single band at 6 kD. In the NSD group, CsA treatment for four weeks significantly decreased EGF expression (cortex, 81 ± 8 vs. 38 ± 3 ; medulla, 85 ± 9 vs. 73 ± 6 , $P < 0.05$, respectively), and CsA treatment in the LSD group almost completely inhibited EGF expression (cortex, 94 ± 10 vs. 2.0 ± 0.5 ; medulla, 219 ± 24 vs. 14 ± 2.5 , $P < 0.05$, respectively). With a concurrent administration of LSRT, EGF expression was significantly increased in the NSD (vs. 182 ± 17 , $P < 0.05$) and the LSD (vs. 269 ± 19 , $P < 0.05$).

Immunohistochemistry of EGF

Immunostaining of EGF in the VH group showed well-localized immunoreactivity in the distal convoluted tu-

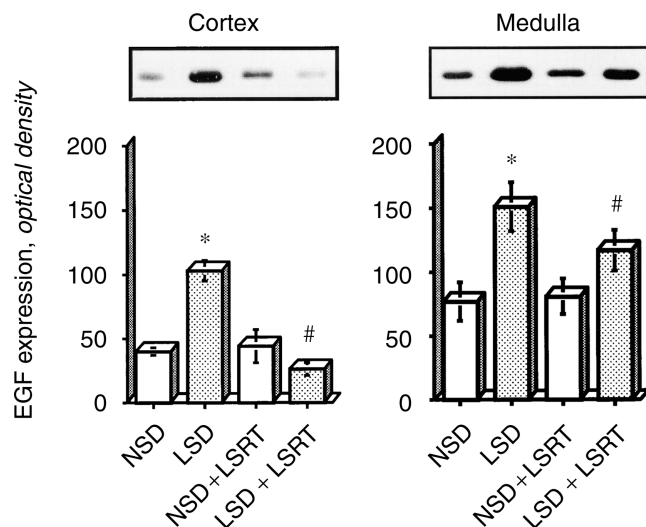


Fig. 7. Immunoblot of epidermal growth factor (EGF) expression in NSD and LSD rats. Note the significant increase of EGF expression in rats on a LSD compared with no significant change in EGF expression in those on a NSD. Concomitant administration of LSRT decreased EGF expression in LSD rats, but no significant change was observed in NSD rats. * $P < 0.05$ vs. NSD; # $P < 0.05$ vs. LSD.

bules and in the thick ascending limb of Henle (Fig. 9A, B). In the CsA group, immunoreactivity of EGF protein was almost undetectable in both renal tubular cells (Fig. 9C and D), but concomitant administration of CsA and LSRT dramatically increased immunoreactivity of EGF in both tubules (Fig. 9E, F).

Relationship between plasma renin activity and EGF expression

The association between RAS with EGF expression was evaluated in normal and CsA-treated rats (Fig. 10). With the LSD, EGF expression was positively correlated with PRA in normal rats (cortex, $r = 0.847$; medulla, $r = 0.899$), but was negatively correlated with PRA in CsA-treated rats (cortex, $r = 0.747$; medulla, $r = 0.722$).

Association between EGF expression and interstitial fibrosis or apoptotic cell death in chronic CsA nephrotoxicity

The association between EGF expression and apoptotic cell death or interstitial fibrosis in chronic CsA nephrotoxicity was evaluated (Fig. 11). Epidermal growth factor expression was well correlated with TIF ($r = 0.664$, $P < 0.01$) and the number of TUNEL-positive cells ($r = 0.822$, $P < 0.01$).

DISCUSSION

The results of our study clearly demonstrate EGF expression in normal and CsA-treated rat kidneys according to the status of RAS. In normal rats, EGF expression was significantly higher in rats on the LSD compared

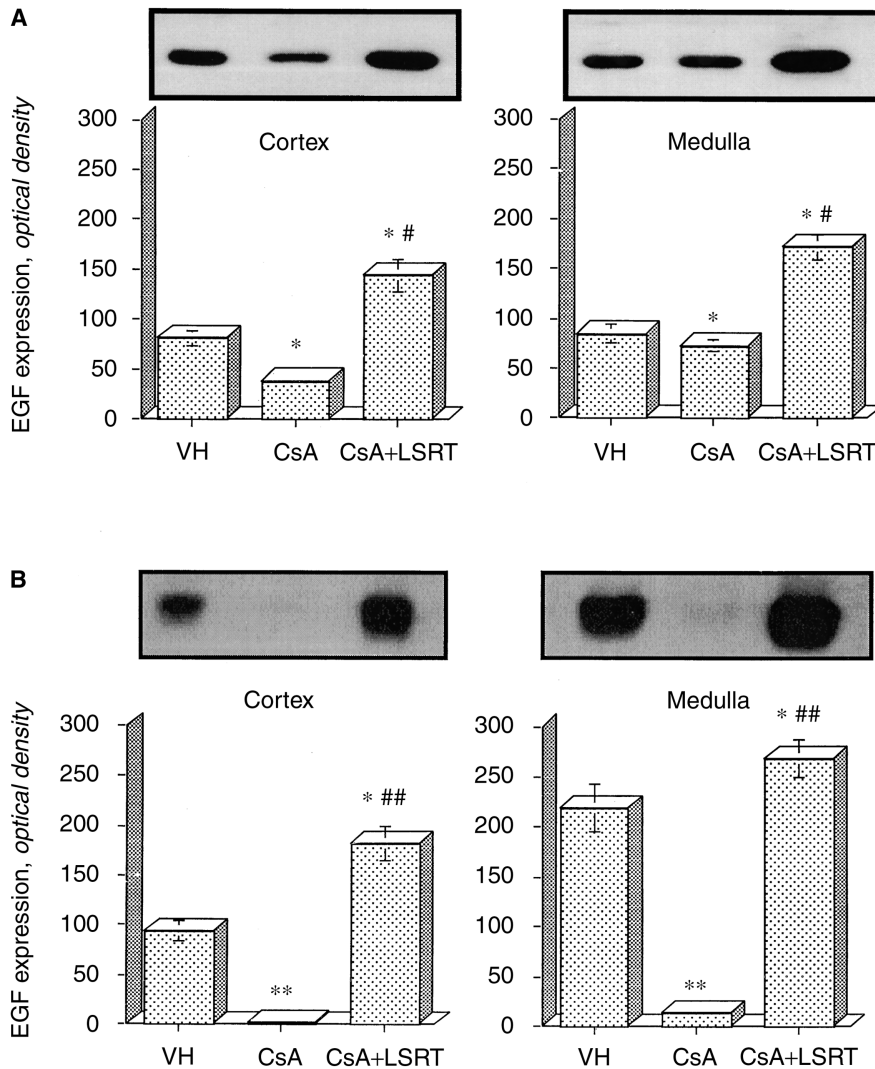


Fig. 8. Immunoblot of EGF in (A) NSD and (B) LSD CsA-treated rats. Note that CsA-treated rats on the LSD almost inhibited EGF expression compared with the rats on the NSD. Administration of LSRT increased EGF expression in rats on the NSD and the LSD, but a greater increase was observed in LSD rats. * $P < 0.05$ vs. VH; ** $P < 0.01$ vs. VH; # $P < 0.05$ vs. CsA; ## $P < 0.01$ vs. CsA.

with the NSD rats, and inhibition of EGF expression by LSRT was observed only in rats on the LSD. On the other hand, CsA-treated rats showed a decreased EGF expression, but Ang II blockade with LSRT preserved the EGF expression, with the effect being more definite in the LSD rats. This finding suggests that EGF expression is influenced by the status of RAS in rat kidney.

The RAS plays an important role in the pathogenesis of the experimental model of chronic CsA nephrotoxicity [7]. CsA administration in salt-depleted rats causes a significant increase in juxtaglomerular apparatus renin, and CsA-induced interstitial fibrosis could be prevented by the use of LSRT or enalapril [8]. Others have demonstrated a beneficial effect with Ang II blockade on CsA-induced afferent arteriopathy and a decrease in the macrophage chemoattractant osteopontin [23]. In the present study, typical chronic CsA nephrotoxicity was observed only in the LSD rats. In addition, CsA with LSD further increased PRA and intrarenal expression of renin com-

pared with the NSD rats. This finding confirms that activation of RAS with an LSD is essential to induce the pathologic lesion of chronic CsA nephrotoxicity and that LSD augments activation of RAS by CsA.

Our study clearly demonstrates that EGF expression in the kidney is regulated by the activity of RAS in normal rats. To define the role of RAS in regulating EGF expression, diets of different sodium contents (NSD vs. LSD) were used. The rats on an LSD showed a significantly increased EGF expression compared with the rats on an NSD (cortex, 2.6-fold; medulla, 1.7-fold). In addition, LSRT treatment significantly decreased the EGF expression in LSD rats, but there was no significant decrease in EGF expression in NSD rats (cortex, 74.8 vs. 10%; medulla, 22.5 vs. 5%). Moreover, EGF expression was well correlated with PRA (cortex, $r = 0.847$; medulla, $r = 0.899$). Based on these observations, we propose that EGF expression in the normal rat kidney is dependent on the activity of RAS.

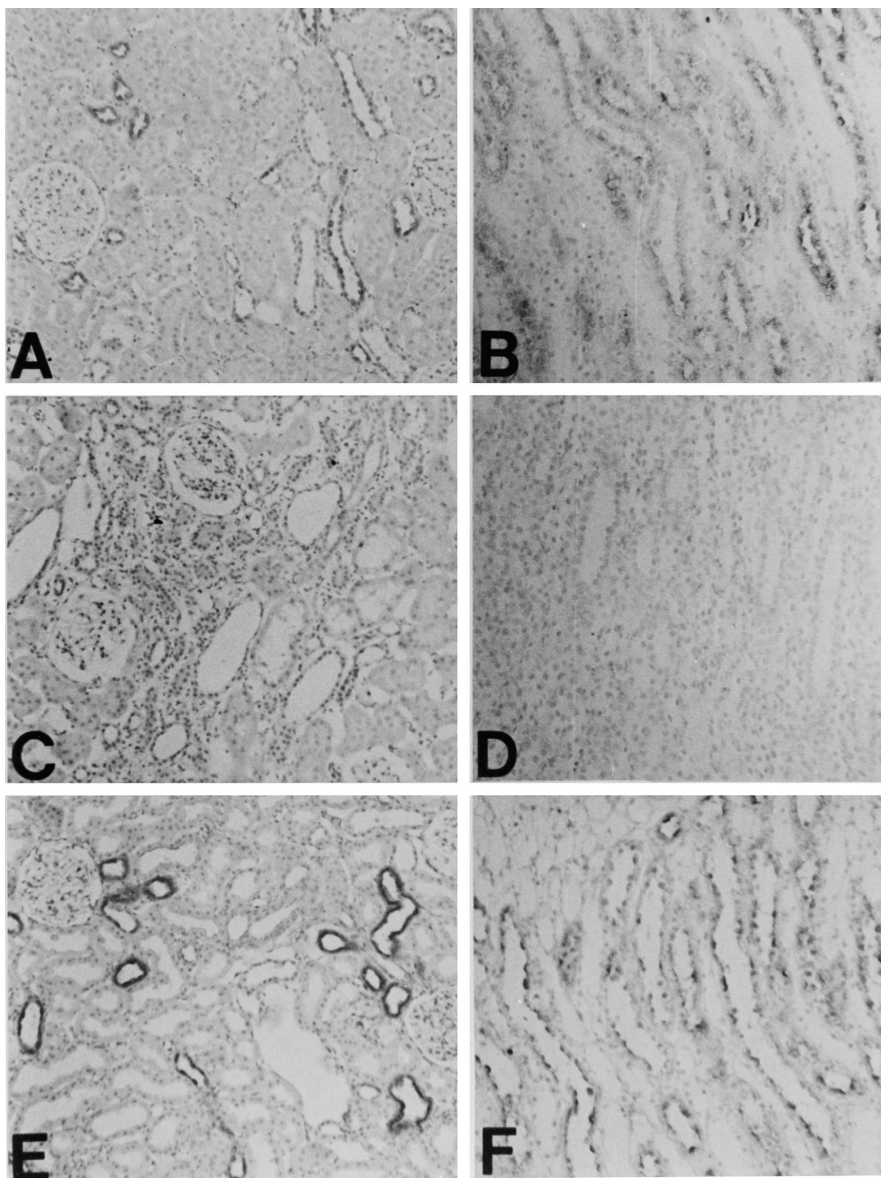


Fig. 9. Immunohistochemistry of EGF in VH (A and B), CsA (C and D), and CsA + LSRT (E and F)-treated groups on the LSD. In the VH group, moderate immunoreactivity of EGF was observed in the distal convoluted tubules (A) and the thick ascending limb of Henle (B). With CsA treatment, the immunoreactivity of two tubules was almost absent (C and D). Concomitant administration of LSRT markedly increased the immunoreactivity of EGF in the distal convoluted tubules (E) and the thick ascending limb of Henle (F). Magnification $\times 200$.

In contrast to rats not receiving CsA treatment, EGF expression decreased in CsA-treated rats on both diets, but a more significant decrease was observed in rats with the LSD compared with the NSD (cortex, 98 vs. 53%; medulla, 94 vs. 14%). The same result was also observed when LSRT and CsA were coadministered. The Ang II blockade significantly increased EGF expression in LSD and NSD rats, but a more significant decrease was observed in rats on the LSD compared with the NSD (cortex, 91- vs. 3.8-fold; medulla, 19- vs. 2.4-fold). In addition, EGF expression was negatively correlated with PRA in rats on the LSD (cortex, $r = 0.749$; medulla, $r = 0.722$). This finding suggests that enhanced CsA-induced nephrotoxicity by LSD overcomes the action of Ang II, which increases EGF expression in normal rats. In addition, it

is conceivable that CsA has a direct toxic effect on EGF expression; the LSD, which enhances the hemodynamic effect of Ang II, might potentiate this effect.

One interesting finding in this study is the overexpression of EGF with the Ang II blockade in CsA-treated NSD rats (cortex, 1.9-fold; medulla, 2.0-fold) or with the LSD (cortex, 2.0-fold; medulla, 1.2-fold) compared with the VH group. This finding suggests that the mechanism of preservation of EGF expression by LSRT is more than that of a reduced CsA-induced nephrotoxicity, and that RAS may be involved in the process of EGF synthesis in CsA-treated rats. In general, EGF is synthesized as a transmembrane prepro-EGF molecule [24, 25], which is a membrane-attached protein with a membrane-spanning hydrophobic domain adjacent to the EGF moiety

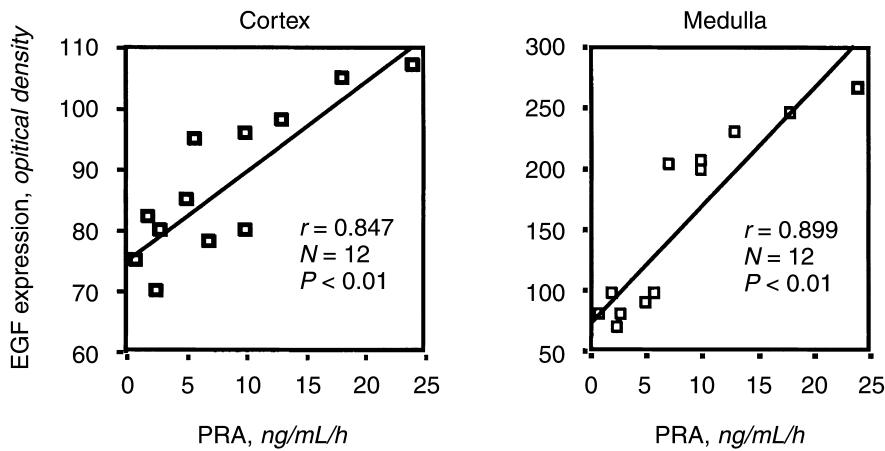
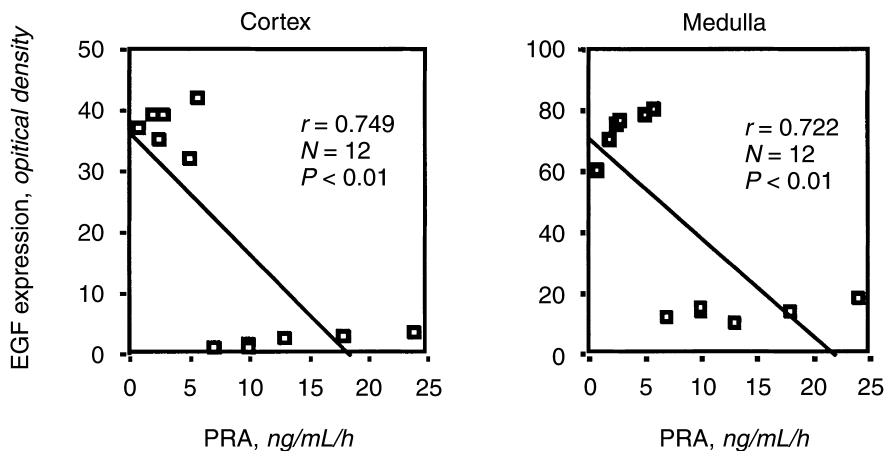
A Normal rats**B CsA-treated rats**

Fig. 10. Association of EGF expression with PRA in (A) normal and (B) CsA-treated rats on the LSD. $N = 12$ and $P < 0.01$ in every group. In A, $r = 0.847$ in cortex and 0.899 in medulla; in B, $r = 0.749$ in cortex and 0.722 in medulla. EGF expression showed a positive correlation with PRA in normal rats, but showed negative correlation with PRA in CsA-treated rats.

[26]. The EGF precursor is not processed to the small peptide in the kidney [27] nor is it easily solubilized from the cell membrane during the extraction process [28]. Based on the previously mentioned findings, we can speculate on some possible mechanisms to explain the overexpression of EGF by LSRT in the CsA-treated rat kidney. First, the Ang II blockade may increase the cleavage of a preformed precursor in CsA-treated rats. Second, the Ang II blockade may inhibit the secretion of a mature form of EGF in CsA-treated rats. Third, there might be a feedback mechanism between RAS and EGF expression in the CsA-treated rat kidney. An *in vitro* study is needed to define the relationship between EGF expression and RAS.

Previous studies have demonstrated that EGF is expressed in the thick ascending limb of Henle and distal convoluted tubules [29–31], which are vulnerable to CsA. In our current study, the VH group showed moderate immunostaining for EGF in the thick ascending limb of

Henle and distal convoluted tubules. With CsA treatment, the intensity of EGF immunoreactivity was almost undetectable in both tubules. In the CsA + LSRT group, the immunoreactivity of EGF was markedly increased in both tubules, which showed more increased immunoreactivity of EGF when compared with the VH group. This immunohistochemical finding is consistent with the immunoblot result and clearly shows the inhibitory effect of CsA and the protective effect of LSRT treatment on EGF expression in chronic CsA nephrotoxicity.

The molecular mechanism of EGF as a growth factor was studied extensively in an experimental model of ischemia/reperfusion injury, and data exist in favor of an involvement of EGF in regenerative hyperplasia after renal injury. Exogenously added EGF has been demonstrated to enhance renal tubule cell regeneration as well as to lessen the severity and duration of acute renal failure after hypoxic injury [32, 33] or exposure to various nephrotoxins such as mercuric chloride [34], folic acid

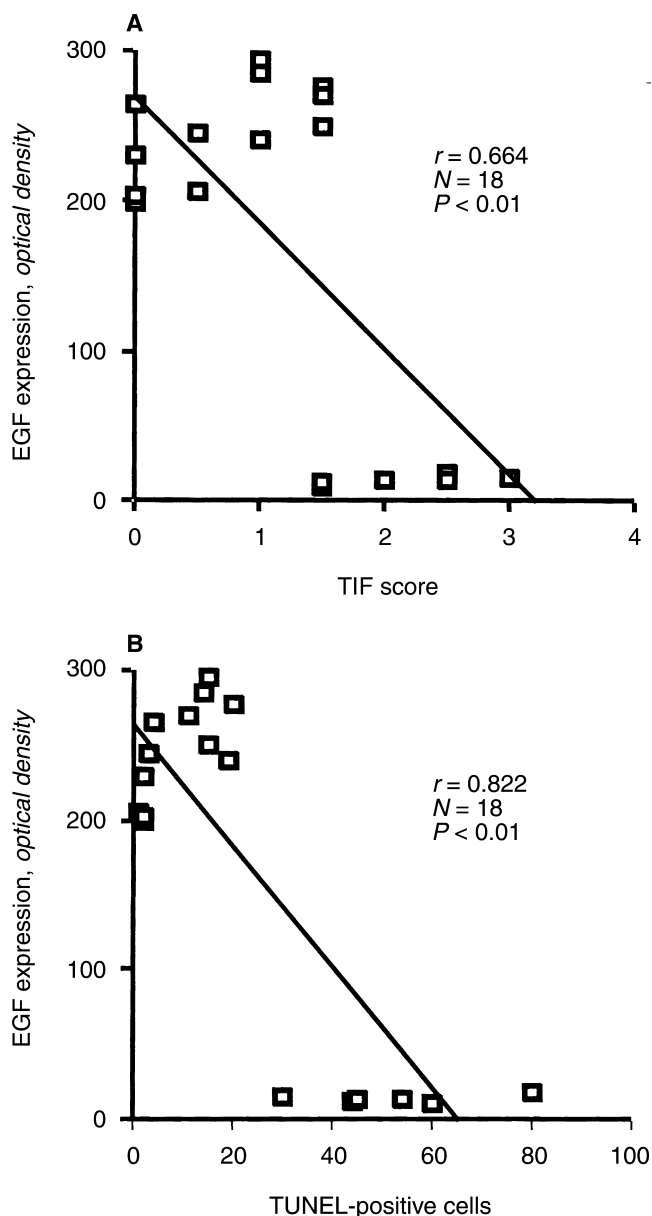


Fig. 11. Association of EGF expression with TUNEL-positive cells (A) or interstitial fibrosis score (B) in CsA-treated rat on a LSD. $N = 12$ and $P < 0.01$ in both groups; $r = 0.664$ in (A) and $r = 0.822$ in (B). EGF expression was well correlated with TUNEL-positive cells or interstitial fibrosis score.

[35], or gentamicin [36]. In the experimental model of chronic CsA nephrotoxicity using salt depletion, interstitial fibrosis [37] and apoptotic cell death [38] are closely associated with activation of RAS, and the blockade of RAS with LSRT or enalapril decreases both parameters [8, 38]. Based on the previously mentioned findings, our investigations were undertaken on the assumption that EGF possibly acts as a survival factor in maintaining tubular cells, and that the loss of EGF might be associated with increased apoptotic cell death or interstitial

fibrosis in CsA-induced nephropathy. Moreover, the protective effect on EGF expression by LSRT might be associated with improvements in both parameters. As expected, EGF expression was well correlated with interstitial fibrosis ($r = 0.664$) and TUNEL-positive cells ($r = 0.822$). This finding suggests that the loss of endogenous EGF during chronic CsA nephrotoxicity is associated with increased apoptotic cell death resulting in interstitial fibrosis. On the other hand, experimental approaches aiming to block EGF receptor signaling seem to ameliorate the evolution of renal diseases. Terzi et al reported a reduction of TIF after renal injury in mice with targeted tubular expression of a dominant negative EGF receptor [39]. This finding suggests that inhibition of EGF signaling in the kidney may prevent the development of renal lesions after renal injury. At present, the fate of EGF receptor in renal tissues exposed to CsA or LSRT is unknown because our experimental investigations focused on EGF itself. However, we suggest that intrarenal EGF may be associated with healing processes, but that it may be helpful or harmful according to the clinical situation. When the kidney is damaged by ischemic or toxic injury, EGF may act as a growth factor in the regeneration of renal tubular cells. In contrast, EGF may have a harmful action in the progression of renal disease in an animal model of uremia (5/6 nephrectomy model).

Our study clearly demonstrates that EGF expression is influenced by the status of RAS in normal and CsA-treated rats. EGF expression is decreased by CsA, but is protected by the Ang II blockade with LSRT. This finding provides a new perspective to the renoprotection of Ang II blockade in cases of chronic CsA nephrotoxicity.

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